# Maternal selenium supplementation and timing of nutrient restriction in pregnant sheep: Effects on maternal endocrine status and placental characteristics[1]

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**ABSTRACT:** To determine the effects of maternal Se intake and plane of nutrition during midgestation, late gestation, or both on hormone and metabolite concentrations in the dam and on placental characteristics, pregnant ewe lambs (n = 64) were assigned to 1 of 8 treatments arranged in a 2 × 2 × 2 factorial array: Se level [initiated at breeding; adequate (3.05 μg/kg of BW) or high (70.4 μg/kg of BW)] and nutritional level [100% (control) or 60% (restricted) of NRC recommendations] fed at different times of gestation [d 50 to 90 (midgestation) or d 91 to 130 (late gestation)]. The control ewes had a greater ($P = 0.01$) percentage change in BW from d 50 than restricted ewes during both mid- and late gestation. Although blood urea N was not affected by either Se or nutritional level, restricted ewes had greater ($P = 0.01$) concentrations of circulating Se on d 66, 78, 106, 120, and 130 of gestation compared with control ewes. Both Se and timing of the nutritional level affected circulating progesterone; however, only nutritional level affected thyroxine and triiodothyronine concentrations in the dam. Nutrient restriction during late gestation decreased ($P \leq 0.01$) fetal BW and fetal fluid weight compared with the control ewes (3.75 vs. 4.13 ± 0.10 kg and 1.61 vs. 2.11 ± 0.11 kg). Although neither Se nor nutritional level affected ($P \geq 0.1$) placental, caruncular, or cotyledonary weights, cotyledonary cellular proliferation was decreased ($P < 0.05$) in ewes receiving a high concentration of Se compared with those receiving adequate Se. In addition, either Se or nutritional level affected vascular endothelial growth factor (VEGFA), VEGFA-receptor 1, VEGFA-receptor 2, and NO synthase mRNA abundance in the cotyledonary tissue. In the caruncular tissue, either Se or nutritional level affected VEGFA-receptor 1, placental growth factor, and NO synthase mRNA abundance. Selenium supplementation and the duration or timing of nutrient restriction appear to influence the endocrine and metabolic status of the ewe, which may influence nutrient transport and placental function.

**Key words:** angiogenic factor, maternal nutrition, placenta, selenium, sheep



J. Anim. Sci. 2010. 88:955–971
doi:10.2527/jas.2009-2152

## INTRODUCTION

Intrauterine growth restriction (**IUGR**) during gestation causes negative effects later in life on animal performance, including postnatal growth, body composition, and reproductive performance (reviewed by Wu et al., 2006). Recently, our laboratories reported that maternal nutrient restriction from d 64 to 135 of gestation in the ewe decreased fetal BW (Reed et al., 2007); however, placentome mass and capillary vascularity were not affected (Lekatz et al., 2009), indicating that altered fetal BW may be due to placental function rather than size. Interestingly, ewes that were given supranutritional amounts (amounts above NRC recommendations) of Se had greater fetal BW compared with ewes receiving adequate amounts of Se (Reed et al., 2007). In addition, increased amounts of Se increased cellular proliferation in the cotyledonary tissue of the placentome (Lekatz et al., 2009). This may indicate that Se provided a sparing effect on fetal BW.

Selenium is an essential trace element because of its important antioxidant capabilities through selenopro-

[1]This project was partially supported by National Research Initiative Competitive Grants 2003-35206-13621 and 2005-35206-15281 from the USDA Cooperative State Research, Education, and Extension Service. Gratitude is expressed to employees of the North Dakota State University Animal Nutrition and Physiology Center and the Reproductive Physiology and Ruminant Nutrition Laboratories for their contributions to this project.

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Received May 21, 2009.
Accepted November 13, 2009.

teins, such as glutathione peroxidase (**GPX**). Preeclamptic women, who often exhibit IUGR, have reduced placental GPX activity compared with control women (Mistry et al., 2008). We have suggested that feeding supplemental Se may increase GPX activity, thereby reducing oxidative stress and sparing fetal BW (Reed et al., 2007; Lekatz et al., 2009). Another selenoprotein, iodothyronine deiodinase, is known to influence the conversion of thyroxine ($\mathbf{T_4}$) to triiodothyronine ($\mathbf{T_3}$; Pappas et al., 2008), which could influence the metabolism of the dam and potentially affect the growth of the fetus and placenta.

In several models of ovine undernutrition during gestation, the results have varied, possibly because of the timing of nutrient restriction (reviewed by Redmer et al., 2004; Luther et al., 2007). Although feed restriction followed by realimentation alters maternal energy metabolism in the pregnant cow (Freetly et al., 2008), it is currently unknown whether maternal restriction followed by realimentation during gestation can influence maternal metabolic status, including such factors as circulating fatty acids in the ewe. Potentially, the endocrine and metabolic status of the dam could influence nutrient availability to the growing fetus for the duration of restriction. Restriction during pregnancy is well noted during times of drought. Moreover, in the upper Midwest, where soils contain elevated concentrations of Se, grazing ruminants could experience increased Se forages along with reduced nutrient intake. The present study was designed to examine the effects of nutrient restriction in 2 distinct periods during gestation (i.e., d 50 to 90 and d 91 to 130). We hypothesized that nutrient restriction would reduce fetal BW, whereas the addition of supranutritional Se to the nutrient-restricted diet would spare fetal BW without affecting placental weight. Moreover, Se status and nutritional level during midgestation, late gestation, or both would alter maternal hormone and metabolite concentrations. The objectives of this study were to determine how supranutritional Se, nutrient restriction, or both during midgestation, late gestation, or both would affect 1) maternal concentrations of circulating NEFA, blood urea N (**BUN**), progesterone, $T_4$, and $T_3$ throughout pregnancy; 2) placental growth and cellularity; and 3) placental angiogenic and vasoactive factor abundance.

## MATERIALS AND METHODS

All procedures were approved by the North Dakota State University and USDA-ARS Animal Care and Use Committees.

### *Animals Management and Treatments*

Western Whiteface ewe lambs originating from the USDA, ARS, US Sheep Experiment Station in Dubois, ID, were synchronized for estrus and exposed to rams for 72 h at the Sheep Experiment Station (Dubois). After breeding (d 0), the rams were removed, and the ewes were assigned randomly to 1 of 2 pens. The pens were assigned randomly to either an adequate Se (**ASe**) or a high Se (**HSe**) dietary treatment. Details of the diets were reported by Carlson et al. (2009). Briefly, ewes were fed a basal diet (2.04 kg/ewe per day; as-fed basis). In addition to the basal diet, at breeding (d 0), ASe ewes were fed 100 g/d (as-fed basis) of a control pellet balanced to contain 0.30 mg of Se/kg to achieve the desired Se intake of 3.05 μg of Se/kg of BW per ewe, whereas HSe ewes were fed 100 g/d of a pellet balanced to contain 47.5 mg of Se/kg, provided as Se-enriched yeast (Sel-Plex, Alltech, Nicholasville, KY), to achieve the desired Se intake of 70.4 μg of Se/kg of BW per ewe.

Pregnancy was determined by ultrasonography (Aloka, Wallingford, CT) in each ewe on d 32 after breeding. Sixty-four ewes (50.7 ± 2.8 kg of BW) were selected to remain on either the ASe (n = 32) or HSe (n = 32) treatment until the end of the experiment, and were transported (1,540 km; 14 h) on d 40 of gestation to the Animal Nutrition and Physiology Center at North Dakota State University (Fargo), where they were housed in individual pens (0.91 × 1.2 m) in an indoor facility until necropsy at d 132 ± 0.9 of gestation. Within the facility, the temperature was held constant at 12°C, and lighting was controlled automatically to mimic the photoperiod of the outdoor environment (from mid January to early May). All ewes had access to fresh water and trace mineralized salt that contained no added Se (American Stockman, Overland Park, KS).

On d 50 of gestation, ewes within each Se treatment were stratified by average breeding date and assigned, along the strata, to receive either 100% (**CON**) or 60% (**RES**) of their nutrient requirements (NRC, 1985) from d 50 to 90 of gestation (i.e., midgestation). On d 91 of gestation, ewes were randomized and reassigned to receive either 100% (CON) or 60% (RES) of their nutrient requirements (NRC, 1985) from d 91 to 130 (i.e., late gestation). Ultimately, this resulted in 8 treatment combinations designated by the following: ASe-CON-CON (control from d 50 to 130; n = 9), ASe-CON-RES (control from d 50 to 90 and restricted from d 91 to 130; n = 7), ASe-RES-CON (restricted from d 50 to 90 and control from d 91 to 130; n = 8), ASe-RES-RES (restricted from d 50 to 130; n = 8), and HSe-CON-CON (control from d 50 to 130; n = 8), HSe-CON-RES (control from d 50 to 90 and restricted from d 91 to 130; n = 8), HSe-RES-CON (restricted from d 50 to 90 and control from d 91 to 130; n = 8), and HSe-RES-RES (restricted from d 50 to 130; n = 8).

During mid- and late gestation, ewes assigned to the ASe treatment derived all dietary nutrients from the control pellet. The HSe ewes were fed the high-Se pellet at a rate that met the desired Se intake (70.4 μg of Se/kg of BW), and the remainder of the diet was composed of the control pellet to achieve the desired ME intake. Beginning on d 50, ewes were weighed every 7 d, and feed intakes of the control and high-Se pellets were adjusted every 2 wk based on ewe BW and stage

of gestation. This approach allowed dietary Se intake to be held constant relative to BW for HSe ewes but for Se intake to vary with DMI for ASe ewes. The percentage change in ewe BW was calculated every 7 d. Ewe blood samples were obtained every 14 d from the jugular vein.

### *Necropsy Procedures*

Ewes were killed on d 132 of gestation (range: d 129 to 136). On the morning of necropsy, ewes were weighed to determine their final BW. Jugular blood samples (10 mL) were collected into both a sterile evacuated nonheparinized tube and a sterile evacuated tube containing 1.8 mg of EDTA per mL of blood (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Serum and plasma were obtained by centrifugation (1,500 × *g* for 30 min at 4°C) and stored at −20°C until further analysis. Exactly 1 h before necropsy, ewes were injected with 5-bromo-2-deoxy-uridine (5 mg/kg of BW) via jugular venipuncture to evaluate cellular proliferation in the placentome. Each ewe was stunned by captive bolt (Supercash Mark 2, Accles and Shelvoke Ltd., Sutton Coldfield, West Midlands, UK) and exsanguinated. The maternal liver was removed and weighed after removing the gall bladder. The gravid uterus was immediately dissected cranial to the cervix and weighed. The uterus was opened along the antimesometrial side, and the umbilical cord was ligated and the fetus was removed and weighed.

Immediately after the fetus was removed, several placentomes were removed from the uterus and weighed. The caruncular and cotyledonary tissues were separated, weighed, snap-frozen, and stored at −80°C. Next, a portion of the gravid uterus was perfusion-fixed according to the methods described by Borowicz et al. (2007). Briefly, the main uterine arterial branch and a main umbilical arterial branch were catheterized, and several placentomes were perfused with PBS followed by Carnoy's solution (a nonaldehyde-based fixative composed of 60% ethanol, 30% chloroform, and 10% glacial acetic acid). Thereafter, the tissues were immersion-fixed in Carnoy's fixative for 24 h. Next, all remaining placentomes were removed from the gravid uterus and weighed. Fetal fluid weight (i.e., combination of amnionic and allantoic fluid) was estimated by subtracting fetal weight, total placentome weight, fetal membrane (interplacentomal membrane) weight, and empty uterine weight from gravid uterine weight.

### *Measurement of Placentome Cellular Proliferation*

After caruncular and cotyledonary tissues were fixed in Carnoy's fixative, they were transferred to a graded series of ethanol solutions, embedded in paraffin, sectioned (4 μm), and mounted on glass slides by using standard histological techniques (Luna, 1968). The prepared placental tissue samples were incubated with an anti-5-bromo-2-deoxy-uridine, formalin grade mouse IgG monoclonal antibody (Clone BMC 9318, Roche Diagnostics, Indianapolis, IN) at a 1:200 dilution (9 μL/1.8 mL) in blocking buffer. Primary antibody was detected using 3,3′-diaminobenzidine (**DAB**; Vector Laboratories, Burlingame, CA), thereby staining the proliferating cells that were in the S (DNA synthetic)-phase of the cell cycle. Hematoxylin (EMD Chemicals Inc., Gibbstown, NJ) was used to counterstain the nondividing nuclei, and periodic acid Schiff's reagent (Luna, 1968) was used to highlight other structures present within the placental tissue cross-section. Microphotographs were taken at 40× magnification with a Nikon DSM 1200 digital camera (Fryer Company Inc., Chicago, IL). Cellular proliferation was quantified using Image-Pro Plus software (version 5.0, Media Cybernetics, Houston, TX). The percentage of proliferating cells was estimated by dividing the number of DAB-stained nuclei by the total number of nuclei (DAB plus hematoxylin stained) present within the area of tissue analyzed.

### *Cellularity Estimates*

Freshly thawed caruncular and cotyledonary samples (0.5 g) were homogenized, using a Polytron instrument with a PT-10s probe (Brinkmann, Westbury, NY), in Tris aminomethane, sodium, and EDTA buffer (0.05 *M* Tris, 2.0 *M* NaCl, 2 m*M* EDTA, pH 7.4). The caruncular and cotyledonary samples were analyzed for DNA, RNA, and protein concentrations. The DNA and RNA analyses were done using the diphenylamine (Johnson et al., 1997) and orcinol procedures (Reynolds et al., 1990). Protein in tissue homogenates was determined with Coomassie brilliant blue G (Bradford, 1976) with BSA (Fraction V, Sigma Chemical, St. Louis, MO) as the standard (Johnson et al., 1997). The prepared samples were analyzed with a spectrophotometer (Beckman DU 640, Beckman Coulter Inc., Fullerton, CA) and were assessed against concentration curves of known standards. The concentration of DNA was used as an index of hyperplasia, and the protein:DNA and RNA:DNA ratios were used as indexes of hypertrophy and potential cellular protein-synthetic activity, respectively (Swanson et al., 2000, 2008; Scheaffer et al., 2004a; Soto-Navarro et al., 2004).

### *Quantification of mRNA Encoding Angiogenic Factors*

Vascular endothelial growth factor (**VEGFA**), its receptors VEGFAR1 (**FLT1**) and VEGFAR2 (**KDR**), placental growth factor (**PGF**), endothelial NO synthase (**NOS3**), and the primary NO receptor soluble guanylate cyclase (**GUCY1A2**) are the major angiogenic and vasoactive factors that have been shown to influence placental vasculature (Redmer et al., 2005; Borowicz et al., 2007; Vonnahme et al., 2007, 2008a,b; Reynolds et al., 2009). These mRNA encoding angio-

genic and vasoactive factors were quantified in caruncular and cotyledonary tissue samples by extracting the total RNA using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Abundance of mRNA for *VEGFA*, *FLT1*, *KDR*, *PGF*, *NOS3*, and *GUCY1A2* were determined by real-time reverse transcription PCR via capillary electrophoresis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE), using methods from our laboratory (Redmer et al., 2005; Vonnahme et al., 2006, 2007; Borowicz et al., 2007) with the following modifications. For each gene of interest, a multiplex reaction was performed by using probe-primer sets for the gene of interest and 18S in each well, as described previously (Vonnahme et al., 2008a). The ratio of the mRNA of the gene of interest to 18S was used for quantifying the gene expression.

### *NEFA and BUN Analysis*

The acyl-CoA synthetase–acyl-CoA oxidase method (NEFA HR, Wako Pure Chemical Industries, Dallas, TX) was used to measure maternal serum NEFA in duplicate. The maternal intraassay and interassay CV were 5.62 and 4.26%, respectively. Maternal serum urea N was measured in duplicate using a urea N kit (Procedure No. 640, Sigma Diagnostics, St. Louis, MO). The maternal intraassay and interassay CV were 4.64 and 3.78%, respectively.

### *Hormone Analysis*

Progesterone was analyzed as described previously (Galbreath et al., 2008). Briefly, a 50-μL sample of maternal serum was analyzed in duplicate. Progesterone concentrations were measured by chemiluminescence immunoassay using an Immulite 1000 immunoassay system (Siemens, Los Angeles, CA), by which lesser, medium, and greater progesterone pools were assayed in duplicate. The intraassay and interassay CV were 6.42 and 9.26%, respectively. Thyroxine and $T_3$ concentrations were determined by chemiluminescence immunoassay using the Immulite 1000 system, using components of commercial kits (Diagnostic Products Corp., Los Angeles, CA) as described previously (O'Neil et al., 2009). Within each assay, lesser, medium, and greater $T_3$ and $T_4$ pools were assayed in duplicate. Twenty-five-microliter and 15-μL serum samples were assayed in duplicate for $T_3$ and $T_4$, respectively. The intraassay CV were 4.27 and 5.87% for $T_4$ and $T_3$, respectively, and the interassay CV were 11.73 and 10.74% for $T_4$ and $T_3$, respectively. In addition, the $T_4$:$T_3$ ratio was calculated.

### *Se and GPX Analysis*

Maternal plasma, caruncular, and cotyledonary samples were analyzed for Se concentration according to the procedures described by Finley et al. (1996). Briefly, 0.25 to 0.30 g of caruncular and cotyledonary tissues were weighed in triplicate into a beaker and 0.5 mL of plasma was measured in triplicate. Within each assay, the samples, the lesser, medium, and greater standards, and blanks were also weighed in triplicate. Samples were placed on a hot plate, and 10 mL of 40% magnesium nitrate solution, 2 mL of 6 *M* HCl, and 10 mL of 16 *M* $HNO_3$ were added to each sample, and the samples were covered and incubated on the hot plate overnight. The covers were removed, and the samples were evaporated to dryness and then ashed at 470°C for 12 to 16 h. After cooling, 10 mL of 12 *M* HCl was added, and the sample was heated on a hot plate for 15 min. When dissolved, the samples were poured into 25-mL volumetric flasks, diluted to volume with double-distilled water, and poured into scintillation vials. Selenium was analyzed with an atomic absorption spectrophotometer with a hydride generator. The intraassay and interassay CV for maternal plasma Se concentration were 5.02 and 3.77%, respectively. The intraassay and interassay CV for caruncular tissue and cotyledonary tissue were 3.61 and 4.06%, and 4.34 and 2.68%, respectively.

Caruncular and cotyledonary samples were also analyzed for Se-dependent GPX activity according to the procedures reported by Paglia and Valentine (1967). Briefly, homogenized sample (20 μL), 15 μL of 0.12 *M* phosphate buffer, and 200 μL of reaction mixture [reduced NAD phosphate (N-6505, Sigma Diagnostics), reduced glutathione (G-4251, Sigma Diagnostics), glutathione reductase (G-4751, Sigma Diagnostics), and phosphate buffer (0.12 *M*)] were added to each well in triplicate. Three wells were filled with 35 μL of buffer solution only, as a blank. Thirty-three microliters of $H_2O_2$ (16.5%) was added to each well. Two plates (96-microwell plates, 260844, Nunc Int., Rochester, NY) for each tissue were used, and absorbance was read at 340 nm (SpectraMax 340, Beckman Coulter) for 4 min at 15-s intervals to measure the oxidation of reduced NAD phosphate to NAD phosphate. With the protein concentration for each sample, the specific activity of GPX was calculated. The intraassay and interassay CV for caruncular and cotyledonary samples averaged 4.73 and 3.61%, and 3.94 and 10.24%, respectively.

### *Statistical Analysis*

Data were arranged in a 2 × 2 × 2 factorial design and were analyzed as a completely randomized design using either the GLM or MIXED procedure (SAS Inst. Inc., Cary, NC). Factors were level of dietary Se (ASe vs. HSe), plane of nutrition during midgestation (CON vs. RES), and plane of nutrition during late gestation (CON vs. RES). The interactions of Se and plane of nutrition during either mid- or late gestation and the 3-way interaction were also included in the model. The 3-way interaction (Se × midgestation nutritional level × late gestation nutritional level) was retained in the model if $P \leq 0.10$; otherwise, the interaction was dropped from the model and all other interactions and main effects were analyzed. The covariate of number of

fetuses carried by each ewe was included in the model for all variables and was retained in the model if $P \leq 0.10$. The main effects of treatments and interactions were deemed significant at $P \leq 0.05$. Means were separated by LSD. The least squares means ± pooled SE are presented. Only significant interactions or main effects are presented. If an interaction or main effect is not presented, it was not significant ($P > 0.05$).

The GLM procedure (SAS Inst. Inc.) was used to analyze ewe liver mass, fetal BW, placental weight, caruncular and cotyledonary weights, placental cellularity, placental angiogenic and vasoactive abundance, and Se and GPX concentrations in the ewe and placenta. The MIXED procedure (SAS Inst. Inc.) was used to analyze ewe BW and Se, NEFA, BUN, progesterone, $T_4$, and $T_3$ concentrations throughout gestation. For the MIXED model, day was included in the analysis for all interactions and as a main effect.

## RESULTS AND DISCUSSION

After transport on d 40 until tissue collection, 9 ewes experienced pregnancy loss, resulting in 55 ewes in the experiment [ASe-CON-CON (n = 9), ASe-CON-RES (n = 5), ASe-RES-CON (n = 3), ASe-RES-RES (n = 7), HSe-CON-CON (n = 7), HSe-CON-RES (n = 8), HSe-RES-CON (n = 8), HSe-RES-RES (n = 8)]. Although it is interesting that there were more pregnancy losses among the ASe ewes, we cannot definitely conclude that the pregnancy loss was due to treatment because this study was not designed to investigate the role of Se on pregnancy retention. Selenium supplementation in dairy cattle (Rutigliano et al., 2008) or ewes (Panter et al., 1995) did not influence pregnancy loss.

### *Percentage Change in Ewe BW Throughout Gestation*

There was no effect of Se on ewe BW gain throughout the experiment; however, nutritional level did affect the percentage of ewe BW gain (Figure 1). As designed, during midgestation (d 66, 73, 80, and 87) and late gestation (d 94, 101, 108, 115, 122, 129, and 136), CON ewes had a greater percentage of BW gain compared with RES ewes ($P = 0.01$; Figure 1). When looking at the overall percentage of BW gain throughout the experiment {100 × [(BW on d 130 − BW on d 50)/BW on d 50]}, there was a Se × midgestation nutritional level × late gestation nutritional level interaction (Figure 2). In the ASe ewes, CON-CON ewes had the greatest percentage of BW gain, RES-CON ewes had a greater ($P = 0.02$; Figure 2) percentage of BW gain compared with CON-RES and RES-RES ewes, and RES-RES ewes had the least BW gain. When ewes were fed HSe, CON-CON ewes had the greatest percentage of BW gain, followed by CON-RES and RES-CON ewes ($P = 0.49$), which did not differ, and RES-RES ewes had the least BW gain. There were similar changes in BW gain for ASe-CON-CON ewes and HSe-CON-CON ewes ($P = 0.65$; Figure 2). The HSe-CON-RES and HSe-RES-CON ewes had a greater ($P = 0.02$; Figure 2) percentage of BW gain compared with ASe-CON-RES and ASe-RES-CON ewes. Regardless of Se treatment, RES-RES ewes were similar in percentage of BW gain ($P = 0.28$; Figure 2).

Selenium supplementation has been reported to increase total BW gain and ADG in growing lambs (Kumar et al., 2008), as well as in guinea pigs (Chaudhary et al., 2009), which the authors attributed to an increase in protein utilization with increased Se in the diet. In the current study, it is interesting that when ewes were nutrient restricted during late gestation, Se supplementation (HSe-late gestation-RES) resulted in a greater BW change at slaughter compared with ewes that were not receiving any additional Se (ASe-late gestation-RES). Increased Se also increased the percentage of BW gain at slaughter when ewes were restricted during midgestation (HSe-midgestation-RES) compared with ewes that were not receiving any additional Se (ASe-midgestation-RES). Although we did not directly measure protein utilization, perhaps Se supplementation in these ewes increased their efficiency. Moreover, our laboratory previously reported that when supranutritional Se is fed 21 d before breeding until d 135 of gestation, greater maternal empty BW (BW − total digesta weight) and fetal BW result (Reed et al., 2007). Ewes in the current study and in that of Reed et al. (2007) received Se-enriched yeast, an organic form of Se. Perhaps Se source plays a role, given that Neville et al. (2008) compared the combined effects of a Se-wheat and 2 levels of selenate, an inorganic form of Se, with a control group. Indeed, when evaluating the maternal tissue loads of the Se sources used in the study by Neville et al. (2008), ewes fed Se-wheat had the most efficient Se loading and had increased fetal concentrations, so perhaps an organically bound form of Se is incorporated into tissue more easily (Taylor et al., 2009). In addition to source, diet composition may play a role in Se absorption because Koenig et al. (1997) found that Se absorption and retention were greater in sheep receiving a concentrate-based diet compared with sheep receiving a forage-based diet. Neville et al. (2008) supplemented a forage-based diet with the Se source, whereas the current study used a pelleted diet.

### *Maternal Plasma Se Concentration Throughout Gestation*

Nutritional level did not affect ($P \geq 0.08$) maternal plasma Se concentration throughout gestation, but there was a Se × day interaction. As anticipated, HSe ewes had a greater ($P = 0.01$) plasma Se concentration on d 50 through 130 of gestation compared with ASe ewes (Figure 3). The greater plasma Se concentration in HSe ewes compared with ASe ewes indicates that Se supplementation effectively elevated the plasma Se concentration, as has been shown in other studies (Reed et al., 2007; Swanson et al., 2008).

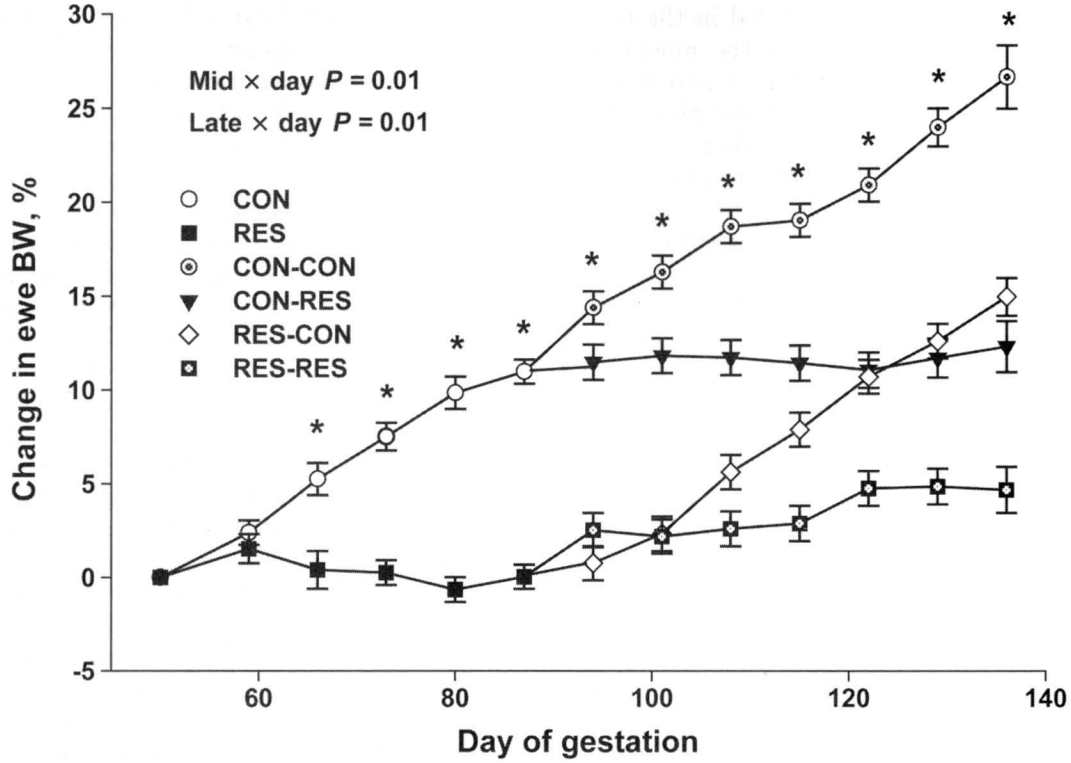


**Figure 1.** Midgestation × day and late gestation × day interactions on percentage of ewe BW gain from d 50 of gestation throughout gestation. Midgestation nutritional treatments were applied from d 50 to 90 of gestation. Late gestation nutritional treatments were applied from d 91 to 130 of gestation. Control (CON) = 100% of nutrient requirements; restricted (RES) = 60% of requirements. An asterisk (*) indicates ewes in the CON group had a greater percentage of BW gain compared with ewes in the RES group.

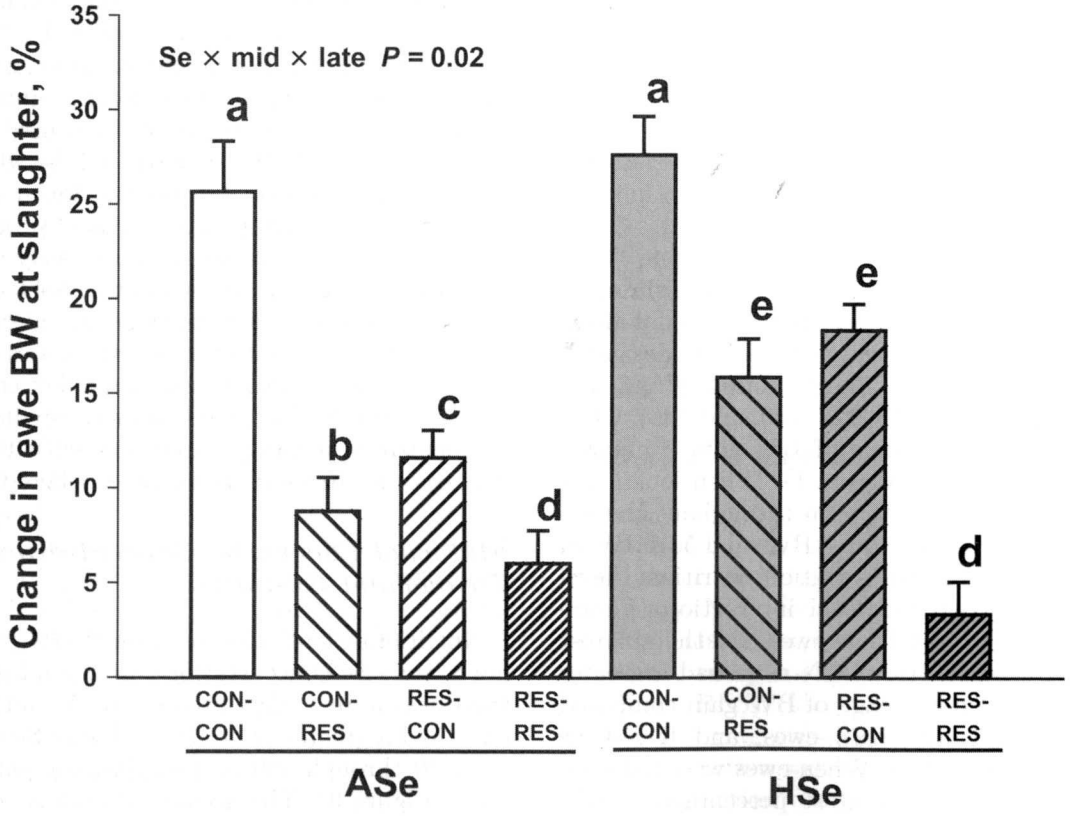


**Figure 2.** The Se × midgestation × late gestation nutritional level interaction on percentage of ewe BW gain from d 50 of gestation to slaughter. Selenium treatments were applied from breeding until slaughter (d 130 of gestation). Adequate Se (ASe) = 3.05 μg/kg of BW; high Se (HSe) = 70.4 μg/kg of BW. Midgestation nutritional treatments were applied from d 50 to 90 of gestation. Late gestation nutritional treatments were applied from d 91 to 130 of gestation. Control (CON) = 100% of nutrient requirements; restricted (RES) = 60% of requirements. Means with different letters (a–e) differ ($P \leq 0.05$).

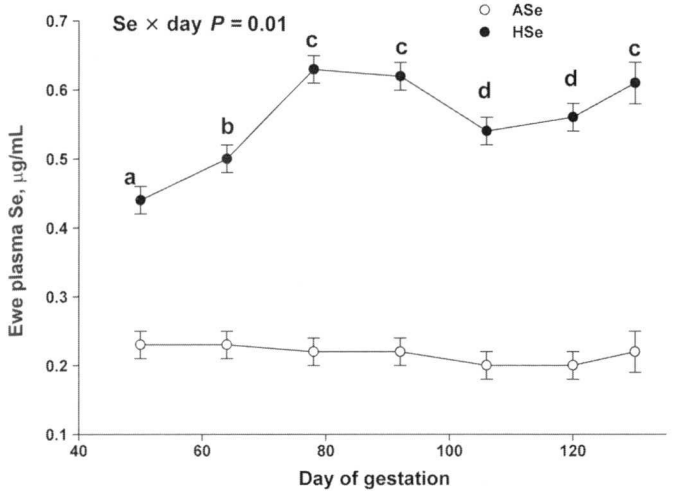


**Figure 3.** Maternal plasma Se concentration throughout gestation. Selenium treatments were applied from breeding until slaughter (d 130 of gestation). Adequate Se (ASe) = 3.05 µg/kg of BW; high Se (HSe) = 70.4 µg/kg of BW. Ewes in the HSe group had greater plasma Se concentrations compared with ewes in the ASe group from d 50 to 130 of gestation. Within the HSe treatment, means with different letters (a–d) differ ($P \leq 0.05$).

Although Se plasma concentration remained unaltered throughout pregnancy in the ASe ewes, HSe ewes experienced an increase in Se concentration from d 50 to 64 and again from d 64 to 78 of gestation (Figure 3). Plasma Se concentration in HSe ewes was greatest from d 78 to 92 of gestation, and then it decreased slightly between d 92 and 120 of gestation before increasing on d 130 of gestation to levels similar to d 78 and 92 of gestation (Figure 3). Although we can only speculate on the reason for this decline in plasma Se during late pregnancy, we have observed this pattern in a previous study (Taylor et al., 2009). Additional work needs to be done on the incorporation of Se into maternal and fetal tissues throughout pregnancy.

### *Maternal NEFA and BUN Concentrations Throughout Gestation*

Ewes had similar ($P = 0.10$) NEFA concentrations on d 50 of gestation (Figure 4). By d 64 of gestation, circulating NEFA concentration was greater ($P = 0.01$) in RES ewes compared with CON ewes (Figure 4), which continued through d 78 of gestation (Figure 4). Regardless of the midgestation nutritional treatment, ewes fed RES diets from d 91 to 130 of gestation had a greater ($P = 0.01$) NEFA concentration from d 106 until 130 of gestation compared with ewes fed CON diets (Figure 4). The greater maternal NEFA concentrations during mid- and late gestation in RES ewes indicate that RES ewes were mobilizing fat stores in response to nutritional deprivation. Similar results have been documented in nutrient-restricted ewes in other studies (Brameld et al., 2000; McMullen et al., 2005; Quigley et al., 2008). These results were expected because the circulating concentration of NEFA is related to the nutritional state (Gordon and Cherkes, 1956; Fredrickson and Gordon, 1958). Circulating NEFA are important sources of available energy, as indicated by the increase in NEFA during fasting (Gordon and Cherkes, 1956; Fredrickson and Gordon, 1958). Although there was no midgestation nutritional level × late gestation nutritional level × day interaction, it is interesting that when ewes were switched from the RES diet to the CON diet, NEFA concentrations declined and were the least at slaughter. Similarly, when ewes were switched from the CON diet to the RES diet, NEFA concentrations increased and were the greatest at slaughter. This indicates that when the nutritional level is changed, the ability of the body to utilize fat stores for energy responds quickly and dramatically.

Neither Se nor nutritional level affected ($P \geq 0.10$) ewe BUN concentration. However, there was a day effect ($P = 0.05$). Maternal BUN concentrations were similar from d 50 to 92 (7.70 ± 0.20 m*M*), increased on d 106 (8.17 ± 0.27 m*M*), and remained elevated until slaughter (8.13 ± 0.23 m*M*). Hileman et al. (1990) observed that ewe BUN concentrations increased after fasting compared with ewes receiving their normal daily intake, indicating that during nutrient deprivation, the body degrades protein for energy. It is interesting that we did not find a difference in BUN in the current study, because we have recently demonstrated by using ultrasonography that nutrient restriction in ewes decreased loin eye area from mid- to late pregnancy (Meyer et al., 2009). Although it was predicted that BUN would be less in RES ewes, we cannot discount the impact that protein catabolism can have during pregnancy when protein requirements are not met. The current study was designed to observe the effects of a global nutrient restriction (i.e., similar reductions in fat, carbohydrates, and protein). The specific role of different durations of protein restriction needs to be investigated.

### *Maternal Progesterone, $T_3$, and $T_4$ Concentrations Throughout Gestation*

There were Se × midgestation nutritional level and midgestation nutritional level × day interaction effects (Figure 5) on maternal progesterone concentration. To describe the Se × midgestation nutritional level interaction, ASe-CON ewes had the least ($P = 0.01$) progesterone compared with all other groups (6.54 vs. 8.79, 7.77, and 7.65 ± 0.55 ng/mL for the ASe-RES, HSe-CON, and HSe-RES treatments, respectively), which did not differ. Regarding the midgestation nutritional level × day interaction, all ewes had similar ($P = 0.59$) progesterone concentrations on d 50 of gestation. By d 64 of gestation, RES ewes had more ($P = 0.01$) circulating progesterone compared with CON ewes (Figure 5). Progesterone concentrations continued to be greater ($P = 0.01$) in RES ewes compared with CON ewes through d 78 of gestation (Figure 5). Plane of nutrition during late gestation did not affect ($P = 0.24$) maternal progesterone concentration. However, it is interesting

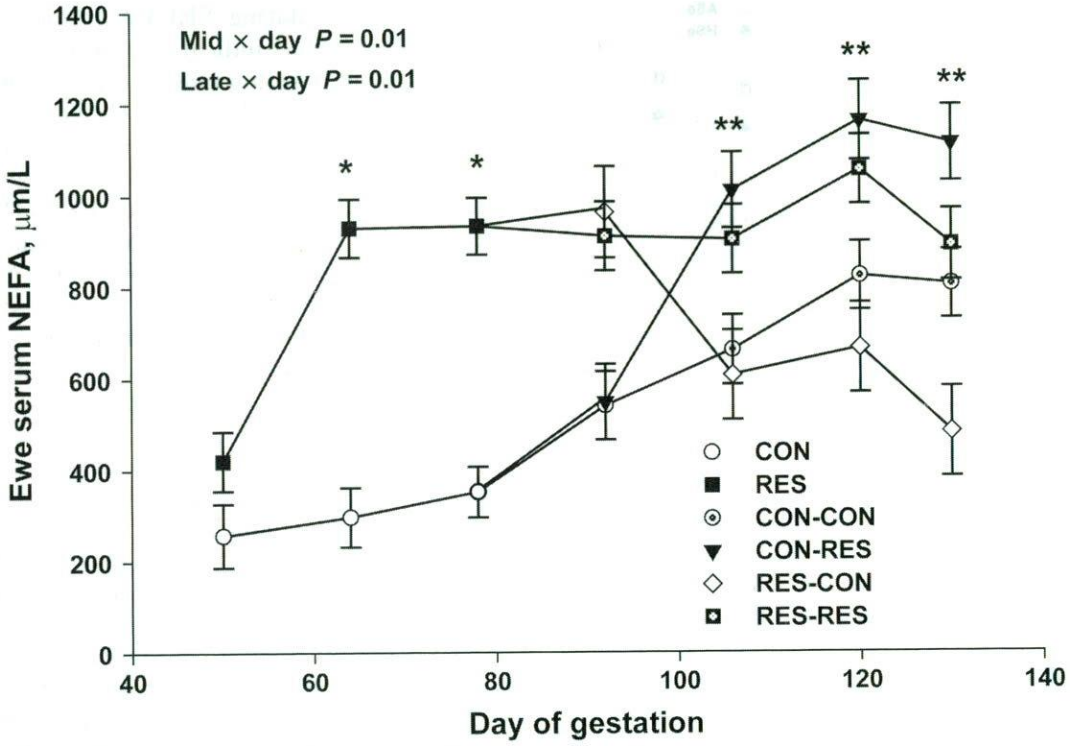


**Figure 4.** Maternal circulating NEFA concentrations throughout gestation. Midgestation nutritional treatments were applied from d 50 to 90 of gestation. Late gestation nutritional treatments were applied from d 91 to 130 of gestation. Control (CON) = 100% of nutrient requirements; restricted (RES) = 60% of requirements. An asterisk (*) indicates ewes in the RES group had an increase ($P \leq 0.05$) in NEFA concentration compared with ewes in the CON group on d 64 and 78 of gestation. A double asterisk (**) indicates ewes in the RES group had an increase ($P \leq 0.05$) in NEFA concentration on d 106, 120, and 130 of gestation.

to note that approximately 2 wk after the late gestational treatments began (from d 92 to 106 of gestation), CON-RES ewes had a 142% increase in circulating progesterone, whereas the other 3 groups (CON-CON, RES-CON, and RES-RES) had an average increase in progesterone of 46.7%. All ewes had similar ($P \geq 0.06$; Figure 5) progesterone concentrations at d 130 of gestation. Lekatz et al. (2009) observed that restricted ewes fed adequate Se had an increase in serum progesterone compared with control ewes fed adequate Se from d 90 to 106 of gestation. Ewes in that study began nutrient restriction on d 64 of gestation, whereas in the current study, nutrient restriction began on d 50 of gestation. It is not known if the increase in progesterone is due to increased production from the placenta or decreased catabolism of progesterone by the liver. However, Freetly and Ferrell (1994) found that progesterone metabolism by splanchnic tissues in the ewe is linearly related to circulating progesterone concentrations and is not influenced by nutritional status. Our differences in progesterone from d 64 through 78 of gestation may be associated with placental growth during this time, as has been reported by McCrabb et al. (1992). Maximal placental growth in the ewe occurs by d 90 (Stegeman, 1974). We may have affected the growth trajectory of the placenta, potentially delaying the peak of placental growth in these RES ewes. Further investigation is needed to verify this hypothesis.

Maternal $T_4$ and $T_3$ concentrations were greater in CON ewes during midgestation compared with RES ewes. There was a midgestation nutritional level × day interaction on maternal $T_4$ concentration (Figure 6A). Concentrations of $T_4$ were greater ($P = 0.01$) in CON ewes compared with RES ewes on d 50, 64, and 78 of gestation (Figure 6A). Similar ($P \geq 0.17$) $T_4$ concentrations were observed on d 92, 106, 120, and 130 of gestation (Figure 6A) regardless of nutritional level.

There were midgestation × late gestation nutritional level and midgestation nutritional level × day (Figure 6B) interactions on ewe $T_3$ concentration. For the midgestation × late gestation nutritional level interaction, CON-CON and CON-RES ewes had greater ($P = 0.01$) maternal $T_3$ concentrations compared with RES-CON and RES-RES ewes (65.06 and 70.79 vs. 57.74 and 56.33 ± 2.97 ng/mL), with CON-CON and CON-RES ewes and RES-CON and RES-RES ewes being similar. For the midgestation nutritional level × day interaction, $T_3$ concentration was greater ($P = 0.03$) in CON ewes compared with RES ewes on d 50, 64, and 78 of gestation (Figure 6B). Concentrations of $T_3$ were similar ($P \geq 0.17$) between CON and RES ewes for the remainder of pregnancy, regardless of the nutritional treatment.

Although neither maternal nutritional level nor Se intake affected ($P \geq 0.06$) the maternal $T_4$:$T_3$ ratio, there was a day effect. The maternal $T_4$:$T_3$ ratio in-

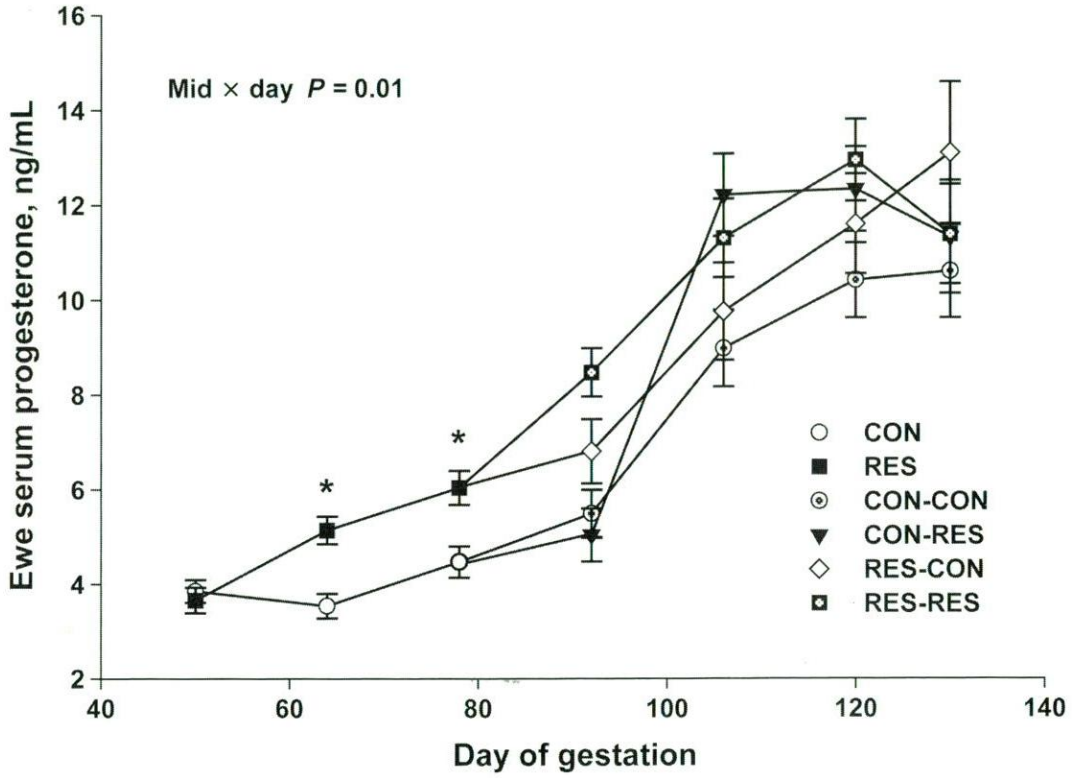


**Figure 5.** Maternal circulating progesterone concentrations throughout gestation. Midgestation nutritional treatments were applied from d 50 to 90 of gestation. Late gestation nutritional treatments were applied from d 91 to 130 of gestation. Control (CON) = 100% of nutrient requirements; restricted (RES) = 60% of requirements. An asterisk (*) indicates ewes in the RES group had an increase ($P \leq 0.05$) in progesterone concentration compared with ewes in the CON group on d 64 and 78 of gestation. See text for details regarding the Se × midgestation nutrition interaction ($P = 0.01$).

creased from d 50 to 64, remaining elevated until d 120 of gestation, when the ratio was similar to that at d 50 (Figure 6C). By d 130, the $T_4$:$T_3$ ratio was greater than at d 50 but was less than at d 106 (Figure 6C).

Elevated concentrations of Se might be expected to alter thyroid hormones, given that Se is important for thyroid metabolism (reviewed in Kohrle et al., 2005); however, no effects of Se were observed on either $T_4$ or $T_3$ concentration or the $T_4$:$T_3$ concentration ratio. Very few studies have evaluated the effects of supranutritional Se on thyroid hormones in sheep. Our results are similar to others who have reported no alteration in $T_4$ or $T_3$ concentrations when supplemental Se was fed to pregnant ewe lambs (Ward et al., 2008) or to growing lambs (Chadio et al., 2006; Kumar et al., 2008). Neither Se nor nutritional level affected the $T_4$:$T_3$ concentration ratio in ewes in the current study. This is in contrast to the results of Ward et al. (2008), who reported that nutrient-restricted ewes provided with adequate Se had the greatest $T_4$:$T_3$ ratio, whereas the increased quantity of Se fed to nutrient-restricted ewes returned $T_4$:$T_3$ ratios to control levels.

### *Necropsy Data*

Gravid uterine, fetal, placentome mass, and estimated fetal fluid weight data are presented in Table 1. Nutrient restriction during late gestation resulted in less ($P \leq 0.01$) gravid uterine mass and estimated fetal fluid weight compared with CON ewes. Furthermore, there was a late gestational effect on fetal BW, with fetuses from RES ewes being lighter ($P = 0.01$) compared with fetuses from CON ewes. These findings are not surprising because numerous studies have shown that nutrient restriction during late gestation leads to decreased fetal BW (Russel et al., 1977; Rattray and Trigg, 1979; Mellor and Murray, 1981; Luther et al., 2007). The reduction in fetal fluid volume could perhaps be attributed to a decreased metabolic rate of the fetus, the placenta, or both, or to alterations in other osmoregulatory factors (Robillard et al., 1992).

Maternal nutrient intake or Se level did not affect total or average placentome weight ($P \geq 0.10$). Further, maternal nutrient intake or Se level had no effect on caruncular or cotyledonary weight ($P \geq 0.45$). This indicates that the observed reduction in fetal BW was not simply due to a reduction in placental mass. Similarly, fetal BW was reduced without a reduction in placental weight when nutrient restriction occurred from d 64 to 135 of gestation in sheep (Reed et al., 2007; Lekatz et al., 2009). Similar to our study, when Luther et al. (2007) compared adolescent ewes that were nutrient restricted for 130 d with a control group, observed reduced fetal BW without a reduction in placental weight. In that study, caruncular capillary area density was decreased in the restricted ewes, whereas we did not make this observation (Lekatz et al., 2009). Neither supplemental Se nor nutrient restriction from d

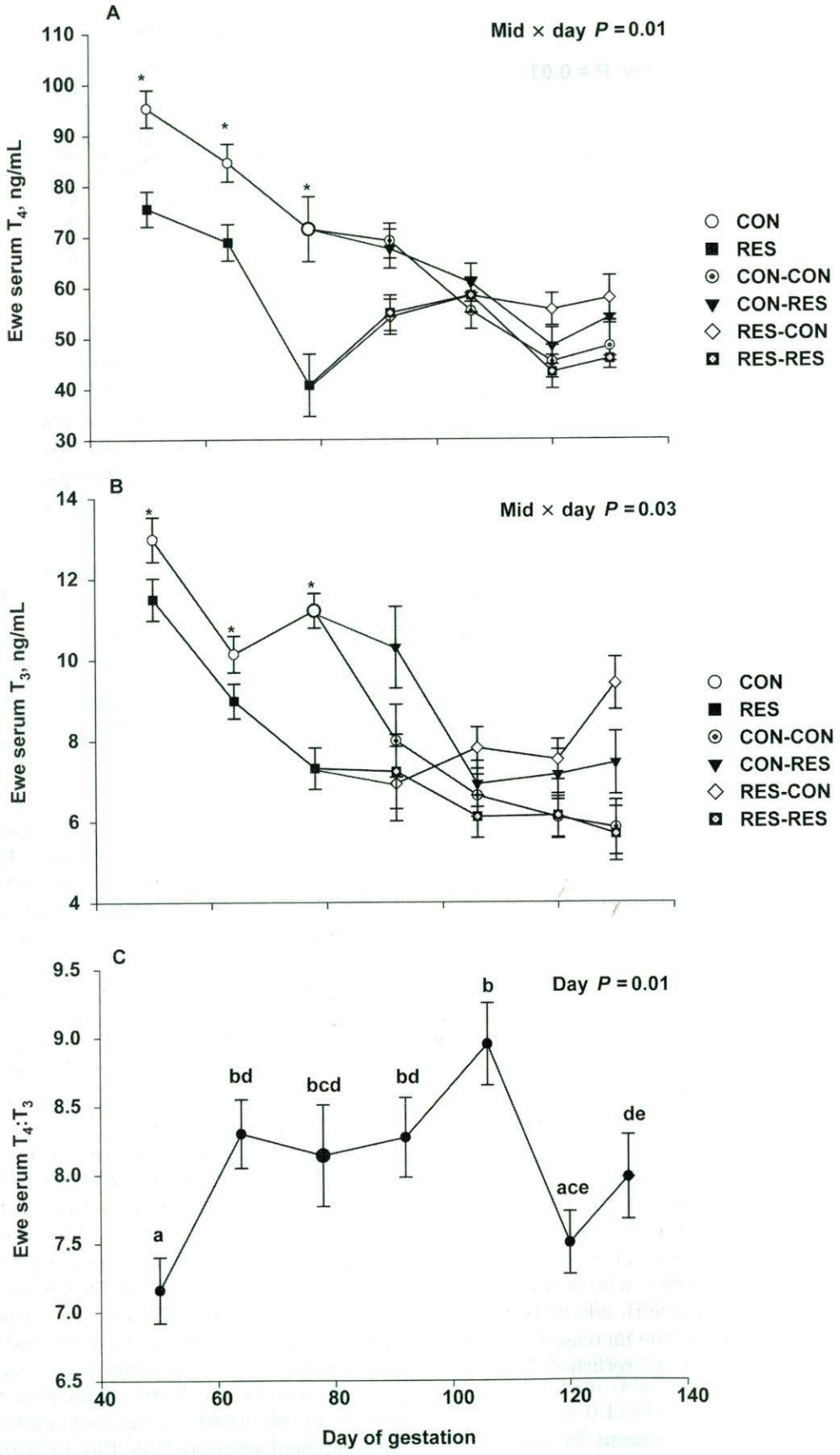


**Figure 6.** Maternal circulating thyroxine ($T_4$; panel A) and triiodothyronine ($T_3$; panel B) concentrations, and the $T_4$:$T_3$ ratio (panel C) throughout gestation. Midgestation nutritional treatments were applied from d 50 to 90 of gestation. Late gestation nutritional treatments were applied from d 91 to 130 of gestation. Control (CON) = 100% of nutrient requirements; restricted (RES) = 60% of requirements. A) For $T_4$, an asterisk (*) indicates midgestation CON ewes had greater $T_4$ concentrations compared with midgestation RES ewes on d 50, 64, and 78 of gestation. B) For $T_3$, an asterisk (*) indicates midgestation CON ewes had greater $T_3$ concentrations compared with midgestation RES ewes on d 50, 64, and 78 of gestation. C) For the $T_4$:$T_3$ ratio, means with different letters (a–e) differ ($P \leq 0.05$).

64 to 130 altered placental capillary vascularity (Lekatz et al., 2009); however, in the study by Luther et al. (2007), no ewes were refed during late pregnancy (i.e., ewes were restricted for the entire duration of the study). Although capillary vascular measurements do provide evidence that the potential nutrient exchange area can be altered in the placenta, this measurement does not account for potential alterations in functional transport capacity. Moreover, the lack of a static measurement near the end of gestation does not explain the angiogenic trajectory of the placentome or the vasoactivity of the uterine arteries, placental arteries, or both. To determine whether the vascular trajectory of the placenta is altered, we will need to investigate placental function at various time points during gestation.

Although there was no interaction of total ewe liver weight or ewe liver weight relative to ewe BW ($P \geq 0.06$), we observed the main effects of Se, midgestation nutritional level, and late gestation nutritional level. To describe the Se effect, ewe liver weight and relative liver weight were lighter ($P = 0.04$ and $P = 0.01$) in ASe ewes compared with HSe ewes (549.0 vs. 580.3 ± 11.18 g/kg of BW, and 9.15 vs. 9.73 ± 0.17 g/kg of BW). There was a midgestation nutritional effect on liver mass, but not on relative liver mass. By the end of gestation, ewes that were provided adequate nutrition during midgestation had greater ($P = 0.01$) liver mass compared with RES ewes (593.1 vs. 536.3 ± 10.94 g). The late gestation nutritional treatment affected both liver mass and relative liver mass. Ewes provided adequate nutrition from d 91 to 130 of gestation had a greater ($P = 0.01$) liver mass (615.1 vs. 514.2 ± 10.57 g) and relative liver mass ($P = 0.01$) compared with RES ewes (9.79 vs. 9.10 ± 0.16 g/kg of BW).

We previously reported that when ewes were restricted from d 50 to 90 of gestation, liver mass was reduced on d 90 compared with control ewes (Scheaffer et al., 2004b). We have demonstrated in the current study that regardless of nutrition during late gestation, nutritional level during midpregnancy affects maternal liver size near term. As energy requirements of ewes (particularly in primiparous ewes that are still growing) increase during pregnancy, nutrient restriction demands that energy resources be prioritized. A good indicator of energy requirements in sheep is the oxygen consumption by the liver. Hepatic oxygen consumption increases with increased feed intake during pregnancy in the ewe (Freetly and Ferrell, 1997), even though liver blood flow does not increase during pregnancy (Rosenfeld, 1977).

### *Placental Cellularity*

In caruncular tissue, there was no effect ($P \geq 0.10$) of either Se or nutritional level on DNA or RNA concentration or on the RNA:DNA ratio, which averaged 1.61 ± 0.15 mg/g, 3.92 ± 0.28 mg/g, and 4.27 ± 1.43, respectively. Likewise, there was no effect of either Se or nutritional level on the percentage of proliferating nuclei in caruncular tissue ($P \geq 0.11$; 0.18 ± 0.02%).

**Table 1.** The effect of Se and nutritional level during mid- and late gestation on fetal mass, placental mass, caruncular mass, cotyledonary mass, and estimated fetal fluid weight

| | Se[1] | | | Midgestation nutrition[2] | | | Late gestation nutrition[3] | | | *P*-value[4] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | ASe | HSe | SE | CON | RES | SE | CON | RES | SE | Se | Midgestation | Late gestation | Se × midgestation × late gestation |
| Gravid uterine wt, kg | 7.28 | 7.22 | 0.33 | 7.26 | 7.24 | 0.32 | 7.89 | 6.61 | 0.31 | 0.88 | 0.96 | 0.01 | 0.70 |
| Fetal mass,[5] kg | 4.02 | 3.86 | 0.11 | 4.03 | 3.85 | 0.11 | 4.13 | 3.75 | 0.10 | 0.29 | 0.21 | 0.01 | 0.13 |
| Total placentome mass, g | 483.40 | 428.61 | 42.57 | 440.89 | 471.11 | 41.63 | 451.08 | 460.93 | 40.23 | 0.33 | 0.60 | 0.86 | 0.61 |
| Average placentome mass,[6] g | 5.17 | 5.65 | 0.54 | 5.41 | 5.41 | 0.52 | 5.49 | 5.33 | 0.51 | 0.51 | 0.99 | 0.82 | 0.55 |
| Caruncular mass, g | 87.83 | 94.82 | 7.17 | 88.44 | 94.21 | 7.01 | 87.26 | 95.40 | 6.78 | 0.47 | 0.55 | 0.39 | 0.52 |
| Cotyledonary mass, g | 291.04 | 288.38 | 20.40 | 287.63 | 291.79 | 19.95 | 299.80 | 279.62 | 19.28 | 0.92 | 0.88 | 0.45 | 0.47 |
| Fetal fluid wt,[7] kg | 1.84 | 1.87 | 0.12 | 1.83 | 1.89 | 0.11 | 2.11 | 1.61 | 0.11 | 0.85 | 0.69 | 0.01 | 0.84 |

[1]Adequate Se (ASe) = 3.05 μg/kg of BW; high Se (HSe) = 70.4 μg/kg of BW. Selenium treatments were applied from breeding until slaughter (d 130 of gestation).
[2]Midgestation nutritional level treatments were applied from d 50 to 90 of gestation. Control (CON) = 100% of energy requirements; restricted (RES) = 60% of energy requirements.
[3]Late gestation nutritional level treatments were applied from d 91 to slaughter (d 130 of gestation). Control (CON) = 100% of energy requirements; restricted (RES) = 60% of energy requirements.
[4]There were no Se × midgestation, Se × late gestation, or midgestation × late gestation nutritional level interactions ($P \geq 0.10$).
[5]A total of 10, 5, 3, and 7 fetuses were collected from ewes in the ASe-CON-CON, ASe-CON-RES, ASe-RES-CON, and ASe-RES-RES treatments, respectively, and 9, 9, 8, and 9 fetuses were collected from ewes in the HSe-CON-CON, HSe-CON-RES, HSe-RES-CON, and HSe-RES-RES treatments, respectively.
[6]Average placentome mass = total placentome mass divided by placentome number.
[7]Estimated fetal fluid weight = gravid uterine weight − (fetal mass + total placentome mass + interplacentome placental tissue mass + empty uterine weight).

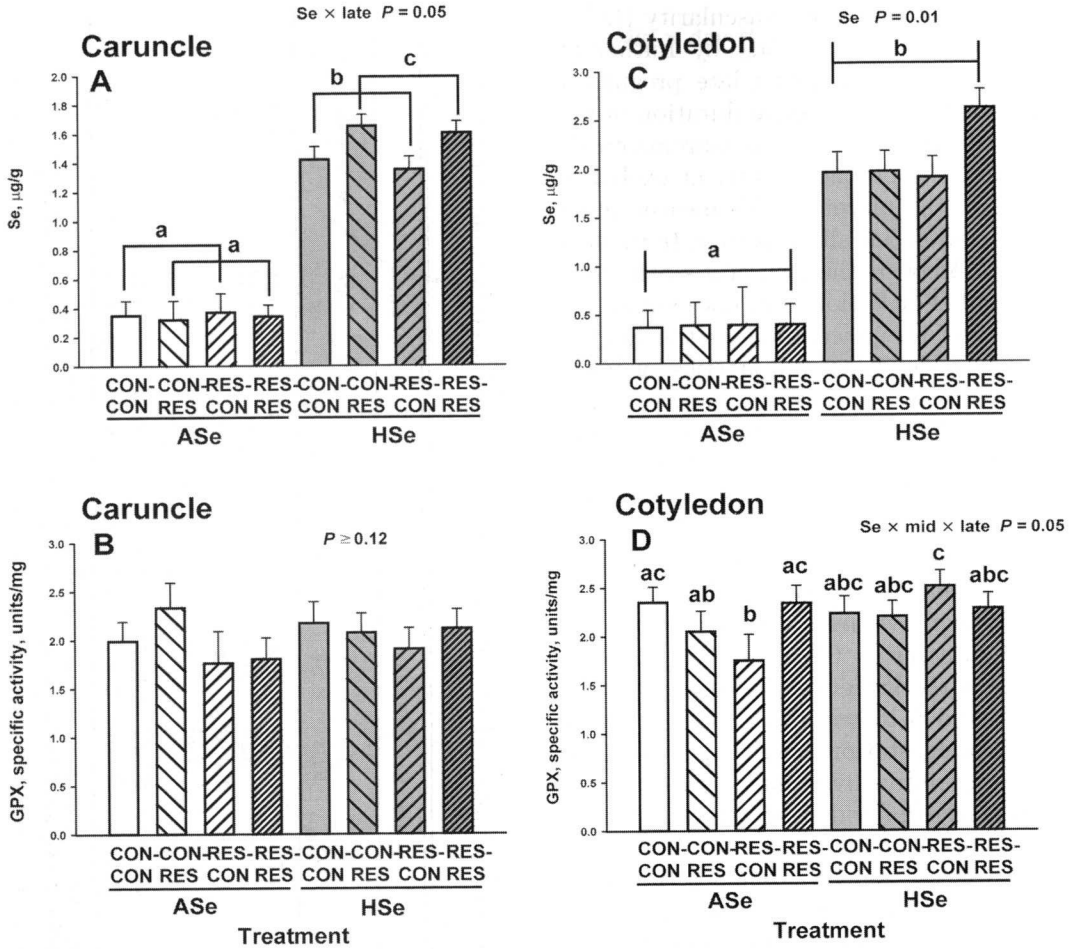


**Figure 7.** The Se × late gestation nutritional level interaction on caruncular Se concentration (panel A), caruncular glutathione peroxidase (GPX) specific activity (panel B), cotyledonary Se concentration (panel C), and cotyledonary GPX specific activity (panel D). Selenium treatments were applied from breeding until slaughter (d 130 of gestation). Adequate Se (ASe) = 3.05 mg/kg of BW; high Se (HSe) = 70.4 mg/kg of BW. Late gestation nutritional treatments were applied from d 91 to 130 of gestation. Control (CON) = 100% of nutrient requirements; restricted (RES) = 60% of requirements. Means with different letters (a–c) differ ($P \leq 0.05$).

There was a Se × midgestation nutritional level interaction on caruncular protein concentration, with ASe-RES ewes having a greater ($P = 0.02$) protein concentration compared with ASe-CON and HSe-RES ewes (120.63 vs. 90.87 and 90.11 ± 12.4 mg/g), with HSe-CON ewes being intermediate (110.83 ± 12.4 mg/g). However, caruncular cell size was not affected by diet ($P \geq 0.13$) because the protein:DNA concentration ratio did not differ.

In cotyledonary tissue, neither Se nor nutritional level affected ($P \geq 0.10$) DNA concentration or the RNA:DNA or protein:DNA ratio, which averaged 2.81 ± 0.19 mg/g, 2.31 ± 0.27, and 45.15 ± 4.02, respectively. However, there was a midgestation × late gestation nutritional level effect on cotyledonary RNA concentration, with RES-CON ewes having a greater ($P = 0.01$) RNA concentration compared with all other groups (7.48 vs. 4.32, 4.72, and 4.47 ± 0.78 mg/g for the RES-CON, CON-CON, CON-RES, and RES-RES groups, respectively). Cotyledonary protein concentration was affected by a Se × midgestation nutritional level interaction. The HSe ewes that were restricted during midpregnancy (d 50 to 90) had less cotyledonary protein compared with ASe-RES and HSe-CON ewes (87.63 vs. 113.17 and 119.72 ± 11.98 mg/g), with ASe-CON ewes being intermediate (96.75 ± 11.98 mg/g) near term. The percentage of proliferating nuclei in the fetal portion of the placenta was affected by Se only. The HSe ewes had a smaller percentage ($P = 0.05$) of proliferating nuclei compared with ASe ewes (1.43 vs. 2.26 ± 0.31%).

Supranutritional Se reduces the incidence of certain types of cancer in humans (Clark et al., 1996). Both increased apoptosis and reduced angiogenesis contribute to inhibiting tumor growth in rats fed supranutritional Se (Combs and Lu, 2001). During early and midpregnancy, the placenta is also a rapidly growing tissue (reviewed in Redmer et al., 2004), and in this study, increased Se decreased cellular proliferation in the cotyledon. However, neither DNA concentration nor cellular size (as indicated by the protein:DNA ratio) or cellular activity (as indicated by the RNA:DNA ratio) in either the caruncular or cotyledonary tissue was affected by Se level. When ewes received supranutritional levels of Se from 21 d before gestation until near term (d 135 of gestation), cotyledonary cellular proliferation

was increased compared with ewes fed adequate levels of Se (Lekatz et al., 2009). In the jejunum, when Se treatments were given 21 d before breeding until d 135 of gestation, there was no effect on the percentage of proliferating nuclei (Reed et al., 2007). Although Se supplementation from breeding until d 130 of gestation did not alter the percentage of proliferating cells in the jejunum, HSe ewes had fewer total proliferating cells in the jejunum compared with ASe ewes (Carlson et al., 2009). It appears that the timing and length of Se treatment may affect how Se alters proliferation in metabolically active tissues such as the placenta and jejunum.

### *Placental Se Concentration and GPX Activity at Necropsy*

In the caruncle, there was a Se × late gestation nutritional level interaction on caruncular Se concentration. Regardless of the nutritional level, ASe ewes had reduced ($P = 0.05$) caruncular Se concentrations compared with HSe ewes (Figure 7A). Furthermore, HSe-RES ewes during late gestation had greater ($P = 0.05$) Se concentrations compared with HSe-CON ewes (Figure 7A; 1.63 vs. 1.39 ± 0.08 mg/g). It has been hypothesized that IUGR pregnancies are further complicated by oxidative stress (Raijmakers et al., 2004; Biri et al., 2007). The greater concentrations of Se in RES ewes may be an adaptation to the stress of nutrient restriction; however, there was no effect ($P \geq 0.12$) of Se or nutritional level on caruncular GPX activity (Figure 7B).

Feeding ewes supplemental Se from breeding until term resulted in HSe ewes having increased ($P = 0.01$) concentrations of cotyledonary Se compared with ASe ewes (Figure 7C; 2.11 vs. 0.33 ± 0.12 mg/g). There was a Se × midgestation nutritional level × late gestation nutritional level interaction on cotyledonary GPX activity (Figure 7D). The ASe-CON-CON and ASe-RES-RES ewes had greater ($P = 0.05$) GPX activity in the cotyledonary tissue compared with ASe-RES-CON ewes, with ASe-CON-RES ewes being intermediate (Figure 7D). Further, HSe-RES-CON ewes had greater ($P = 0.05$) GPX activity compared with ASe-RES-CON ewes. Within HSe ewes, level of nutrition did not affect GPX activity (Figure 7D).

Blood flow can be regulated by the balance between free radical production and the bioavailability of NO (Chen and Keaney, 2004). When there is an increase in reactive oxygen species (**ROS**), in particular $O_2 \cdot^-$, the ROS will react with NO to form peroxynitrite. In addition to ROS directly causing oxidative damage to the cells, there is a reduction in blood flow caused by the inactivation of NO (Schulz et al., 2004). Antioxidant compounds, such as GPX, limit oxidative damage and restore endothelial cell function.

Intrauterine growth restriction, which can be caused by nutrient restriction and reduced uterine blood flow, leads to an increase in oxidative stress (Biri et al., 2007). This increase in oxidative stress is coupled with an increase in placental GPX activity in the IUGR pregnancies compared with normal pregnancies (Biri et al., 2007). Because ASe-RES-RES ewes were restricted for a longer period of time compared with ASe-RES-CON ewes, we hypothesized that there may have been more oxidative stress within the placenta of the ASe-RES-RES ewes, which might explain why these ewes had greater GPX activity.

Although Knapen et al. (1999) and Biri et al. (2007) reported that placental tissue from women with IUGR fetuses had increased GPX activity compared with normal pregnancies, Mistry et al. (2008) reported that placentas from preeclamptic women had reduced GPX activity compared with normal pregnancies. It has been suggested that providing antioxidants during pregnancy could decrease oxidative stress and may therefore prevent IUGR (Biri et al., 2007). Oxidative stress in the placenta is also increased in preeclampsia (Sikkema et al., 2001), and women suffering from preeclampsia have reduced concentrations of Se in umbilical venous blood compared with women with normal pregnancies (Mistry et al., 2008). In the current study, feeding supranutritional Se did not spare fetal BW in the RES fetuses. Reed et al. (2007) found that feeding increased levels of Se increased fetal BW compared with feeding adequate Se, regardless of nutritional level, indicating the potential for increased levels of Se to increase fetal BW. It may be useful to investigate the effects of Se supplementation on both GPX activity and oxidative stress in the placenta to understand how these variables might influence fetal BW.

### *Angiogenic and Vasoactive mRNA Abundance*

In this study, Se, nutritional level, or both affected angiogenic and vasoactive mRNA abundance in both the caruncle and cotyledon. However, the treatments appeared to have affected the caruncular and cotyledonary tissues differently.

In the caruncle, there was no effect of supranutritional Se or plane of nutrition during mid- or late gestation ($P \geq 0.10$) on *VEGFA*, *KDR*, or *GUCY1A2* mRNA abundance. Ewes fed increased levels of Se had less ($P = 0.05$) caruncular *FLT1* mRNA abundance compared with ASe ewes (0.18 vs. 0.27 ± 0.03). There was a Se × late gestation nutritional level interaction on caruncular *NOS3* mRNA abundance, with ASe-CON ewes having greater ($P = 0.04$) *NOS3* mRNA abundance than ASe-RES, HSe-CON, and HSe-RES ewes (Table 2). There was also a Se × late gestation nutritional level interaction on caruncular *PGF* mRNA expression, with ASe-CON ewes having greater ($P = 0.05$) *PGF* mRNA abundance than HSe-CON ewes and ASe-RES and HSe-RES ewes being intermediate (Table 2).

In the cotyledonary tissue, ASe ewes had greater ($P = 0.01$) cotyledonary *VEGFA* mRNA abundance compared with HSe ewes (1.27 vs. 0.86 ± 0.08). There was a Se × late gestation nutritional level interaction on

**Table 2.** The effect of Se and nutritional level during mid- and late gestation on caruncular and cotyledonary vascular endothelial growth factor (*VEGFA*), vascular endothelial growth factor receptor VEGFAR1 (*FLT1*), vascular endothelial growth factor receptor VEGFAR2 (*KDR*), endothelial NO synthase (*NOS3*), primary NO receptor soluble guanylate cyclase (*GUCY1A2*), and placental growth factor (*PGF*) mRNA expression[1]

| | Se[2] × midgestation nutrition[3] | | | | | Se[2] × late gestation nutrition[4] | | | | | Midgestation × late gestation nutrition[3,4] | | | | | *P*-value[5] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ASe | | HSe | | | ASe | | HSe | | | | | | | | | | |
| Item | CON | RES | CON | RES | SE | CON | RES | CON | RES | SE | CON-CON | CON-RES | RES-CON | RES-RES | SE | Se × midgestation | Se × late gestation | Midgestation × late gestation |
| Caruncle | | | | | | | | | | | | | | | | | | |
| *VEGFA* | 0.32 | 0.25 | 0.21 | 0.21 | 0.06 | 0.33 | 0.24 | 0.21 | 0.21 | 0.05 | 0.29 | 0.23 | 0.25 | 0.22 | 0.05 | 0.45 | 0.33 | 0.76 |
| *FLT1*[6] | 0.27 | 0.26 | 0.18 | 0.19 | 0.05 | 0.31 | 0.22 | 0.19 | 0.18 | 0.04 | 0.25 | 0.20 | 0.24 | 0.21 | 0.05 | 0.74 | 0.31 | 0.86 |
| *KDR* | 0.15 | 0.15 | 0.25 | 0.15 | 0.04 | 0.14 | 0.16 | 0.18 | 0.22 | 0.03 | 0.18 | 0.22 | 0.14 | 0.16 | 0.04 | 0.12 | 0.64 | 0.75 |
| *NOS3* | 1.01 | 1.01 | 0.58 | 0.65 | 0.11 | $1.19^{a}$ | $0.83^{b}$ | $0.60^{b}$ | $0.63^{b}$ | 0.10 | 0.80 | 0.78 | 0.98 | 0.67 | 0.10 | 0.71 | 0.04 | 0.12 |
| *GUCY1A2* | 1.58 | 1.39 | 1.11 | 1.33 | 0.23 | 1.39 | 1.58 | 1.39 | 1.04 | 0.23 | 1.42 | 1.27 | 1.37 | 1.35 | 0.23 | 0.30 | 0.20 | 0.75 |
| *PGF* | 0.26 | 0.31 | 0.23 | 0.21 | 0.04 | $0.33^{a}$ | $0.23^{ab}$ | $0.19^{b}$ | $0.25^{ab}$ | 0.04 | 0.23 | 0.26 | 0.29 | 0.22 | 0.04 | 0.32 | 0.05 | 0.22 |
| Cotyledonary | | | | | | | | | | | | | | | | | | |
| *VEGFA*[7] | 1.41 | 1.12 | 0.82 | 0.89 | 0.12 | 1.43 | 1.10 | 0.88 | 0.83 | 0.09 | 1.22 | 1.01 | 1.09 | 0.92 | 0.12 | 0.09 | 0.19 | 0.87 |
| *FLT1* | $1.39^{a}$ | $1.00^{bc}$ | $0.79^{b}$ | $1.01^{c}$ | 0.12 | 1.17 | 1.21 | 0.91 | 0.90 | 0.12 | 1.08 | 1.10 | 1.00 | 1.01 | 0.12 | 0.01 | 0.80 | 0.98 |
| *KDR* | 0.53 | 0.63 | 0.32 | 0.45 | 0.04 | $0.67^{a}$ | $0.49^{b}$ | $0.36^{c}$ | $0.41^{bc}$ | 0.04 | $0.42^{a}$ | $0.44^{a}$ | $0.62^{b}$ | $0.46^{a}$ | 0.04 | 0.71 | 0.01 | 0.02 |
| *NOS3* | $1.15^{a}$ | $0.98^{ab}$ | $0.70^{c}$ | $0.89^{bc}$ | 0.09 | $1.17^{a}$ | $0.96^{b}$ | $0.73^{c}$ | $0.86^{bc}$ | 0.09 | 0.91 | 0.94 | 0.99 | 0.88 | 0.09 | 0.04 | 0.04 | 0.39 |
| *GUCY1A2* | 1.03 | 0.88 | 0.82 | 1.03 | 0.16 | 0.99 | 0.92 | 0.90 | 0.95 | 0.15 | 0.90 | 0.95 | 0.99 | 0.92 | 0.15 | 0.18 | 0.66 | 0.64 |
| *PGF* | 0.94 | 0.84 | 0.83 | 0.73 | 0.15 | 0.99 | 0.79 | 0.69 | 0.87 | 0.14 | 0.79 | 0.98 | 0.89 | 0.68 | 0.14 | 0.99 | 0.14 | 0.11 |

[a–c]Values within a row and category with different superscripts differ ($P \leq 0.05$).

[1]Expressed as a ratio to 18S mRNA expression.

[2]Adequate Se (ASe) = 3.05 μg/kg of BW; high Se (HSe) = 70.4 μg/kg of BW. Selenium treatments were applied from breeding until slaughter (d 130 of gestation).

[3]Midgestation nutritional level treatments were applied from d 50 to 90 of gestation. Control (CON) = 100% of requirements; restricted (RES) = 60% of requirements.

[4]Late gestation nutritional level treatments were applied from d 91 to slaughter (d 130 of gestation). Control (CON) = 100% of requirements; restricted (RES) = 60% of requirements.

[5]There were no Se × midgestation × late gestation nutrition interactions ($P \geq 0.12$) for either caruncular or cotyledonary angiogenic and vasoactive factor mRNA expression.

[6]There was a main effect of Se ($P = 0.05$) on caruncular *FLT1* mRNA expression; ASe = 0.27 vs. HSe = 0.18 ± 0.03.

[7]There was a main effect of Se ($P = 0.01$) on cotyledonary *VEGFA* mRNA expression; ASe = 1.27 vs. HSe = 0.86 ± 0.08.

cotyledonary *KDR* mRNA abundance, with ASe-CON ewes having greater ($P = 0.01$) *KDR* mRNA abundance than ewes in all other groups (Table 2). In addition, HSe-CON ewes had less ($P = 0.01$) cotyledonary *KDR* mRNA abundance compared with ASe-CON and ASe-RES ewes (Table 2), whereas the cotyledonary *KDR* mRNA abundance of HSe-RES ewes was similar to that of both HSe-CON and ASe-RES ewes (Table 2). There was also a midgestation × late gestation nutritional level interaction on cotyledonary *KDR* mRNA abundance. The RES-CON ewes had greater ($P = 0.02$) cotyledonary *KDR* mRNA abundance than ewes of all other groups, which did not differ (Table 2). Cotyledonary *FLT1* mRNA abundance was also affected by a Se × midgestation nutritional level interaction. The ASe-CON ewes had greater ($P = 0.01$) *FLT1* cotyledonary mRNA abundance than ewes of all other groups (Table 2). To further describe the Se × midgestation nutritional level interaction, HSe-CON ewes had less ($P = 0.01$) *FLT1* mRNA expression in the cotyledonary tissue compared with HSe-RES ewes (Table 2). There was a Se × midgestation nutritional level interaction on cotyledonary *NOS3* mRNA abundance. The ASe-CON ewes had greater ($P = 0.04$) cotyledonary *eNOS* mRNA abundance compared with the HSe-CON and HSe-RES ewes (Table 2). Further, ASe-RES ewes had greater ($P = 0.04$) cotyledonary *NOS3* mRNA abundance than HSe-CON ewes (Table 2). The ASe-RES ewes and the HSe-RES ewes had similar cotyledonary *NOS3* mRNA abundance (Table 2), as did the HSe-CON and HSe-RES ewes (Table 2). There was also a Se × late gestation nutritional level interaction on cotyledonary *NOS3* mRNA abundance, with ASe-CON ewes having greater ($P = 0.04$) *NOS3* mRNA abundance than ewes in all other groups (Table 2). The HSe-CON ewes had less ($P = 0.04$) cotyledonary *NOS3* mRNA abundance than ASe-RES ewes (Table 2), whereas the cotyledonary *NOS3* mRNA abundance of HSe-RES ewes was similar to that of both HSe-CON and ASe-RES ewes (Table 2). There were no significant interactions or effects on cotyledonary *GUCY1A2* or *PGF* mRNA abundance (Table 2).

Borowicz et al. (2007) reported that caruncular vascularity in the ewe from mid- to late gestation increases primarily through vasodilatation. This agrees with our findings because *PGF* and *FLT1* mRNA abundance was affected in the caruncular tissue. Osol et al. (2008) observed that placental growth factor binds to *FLT1* and that *PGF* increases the vasodilatation of vessels in vitro. Borowicz et al. (2007) found that whereas the caruncular tissue in the ewe increases via vasodilatation during mid- to late gestation, capillary numbers and the surface area of nutrient exchange increase tremendously during the last half of gestation in the cotyledon. Currently, little information is available on the effects of Se on angiogenic factors in the placenta. There is a wealth of data on decreasing angiogenic factors, vascularity, or endothelial cell proliferation in tumors or in cancer cell lines (Jackson and Combs, 2008; Zeng and Combs, 2008). Collectively, we report that maternal Se supplementation for 130 d decreased angiogenic factor mRNA abundance in the placentomes, regardless of the duration of restriction.

It is important to note that many of the alterations in mRNA abundance in the placenta collected near term were influenced by what the ewe was consuming during midgestation. Vonnahme et al. (2007) reported that in the cow, placental measures for vascularity were not affected immediately after restriction, but were seen after realimentation occurred. This indicates that the growth trajectory of the placenta is affected either by the earlier diet or by the process of realimentation. It would be useful to investigate whether resetting the maternal endocrine status, metabolic status, or both drives the fluctuation in placental dynamics. As mentioned above, Lekatz et al. (2009) demonstrated that when ewes remained restricted throughout mid- to late gestation, there was no alteration in placentome vascularity. Perhaps the trigger for angiogenesis or vasodilatation is the sudden change in maternal metabolic status. How Se is playing a role during this period of realimentation is not yet known.

In summary, this is the first study to investigate the combined effects of Se and nutrient restriction during midgestation, late gestation, or both on maternal hormone and metabolite concentrations throughout pregnancy and placental angiogenic and vasoactive factor mRNA abundance. It is unknown whether supplemental Se could protect the developing placenta or fetus from oxidative stress that may occur during bouts of nutrient restriction. However, it appears that fetal weight can be spared if the maternal system is rescued from the restriction by d 90 of pregnancy in the ewe. It is unknown how this earlier restriction may influence both the maternal and fetal systems. The maternal ability to provide adequate milk during lactation, as well as to have the ability to return to estrus and conceive again, is not known. Further, fetal weight, although a good indicator of neonatal health, does not fully predict the vigor and growth potential of the offspring. An understanding of how the maternal system recovers from nutritional stressors during pregnancy, and perhaps how the placenta adapts to this metabolic shift, is important to the development of healthy offspring.

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